Prolonged Exposure to LPS Increases Iron, Heme, and p22\textsuperscript{phox} Levels and NADPH Oxidase Activity in Human Aortic Endothelial Cells

Inhibition by Desferrioxamine

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Objective—Vascular oxidative stress and inflammation are contributing factors in atherosclerosis. We recently found that the iron chelator, desferrioxamine (DFO), suppresses NADPH oxidase-mediated oxidative stress and expression of cellular adhesion molecules in mice treated with lipopolysaccharide (LPS). The objective of the present study was to investigate whether and how LPS and iron enhance, and DFO inhibits, NADPH oxidase activity in human aortic endothelial cells (HAECs).

Methods and Results—Incubation of HAECs for 24 hours with 5 \(\mu\)g/mL LPS led to a 4-fold increase in NADPH oxidase activity, which was strongly suppressed by pretreatment of the cells for 24 hours with 100 \(\mu\)mol/L DFO. Incubating HAECs with LPS also significantly increased cellular iron and heme levels and mRNA and protein levels of p22\textsuperscript{phox}, a heme-containing, catalytic subunit of NADPH oxidase. All of these effects of LPS on HAECs were strongly inhibited by DFO. Exposing HAECs to 100 \(\mu\)mol/L iron (ferric citrate) for 48 hours exerted similar effects as LPS, and these effects were strongly inhibited by coincubation with DFO. Furthermore, neither LPS nor DFO affected mRNA and protein levels of p47\textsuperscript{phox}, a nonheme-containing, regulatory subunit of NADPH oxidase, or the mRNA level of NOX4, an isoform of the principal catalytic subunit of NADPH oxidase in endothelial cells. In contrast, heme oxygenase-1 was strongly suppressed by DFO, both in the absence and presence of LPS or iron.

Conclusions—Our data indicate that prolonged exposure to LPS or iron increases endothelial NADPH oxidase activity by increasing p22\textsuperscript{phox} gene transcription and cellular levels of iron, heme, and p22\textsuperscript{phox} protein. Iron chelation by DFO effectively suppresses endothelial NADPH oxidase activity, which may be helpful as an adjunct in reducing vascular oxidative stress and inflammation in atherosclerosis. (Arterioscler Thromb Vasc Biol. 2009;29:732-738.)

Key Words: desferrioxamine ■ iron ■ lipopolysaccharide ■ NADPH oxidase ■ NOX4 ■ p22\textsuperscript{phox} ■ p47\textsuperscript{phox}

Vascular inflammation and oxidative stress play prominent roles in the pathogenesis of atherosclerosis and cardiovascular diseases.\textsuperscript{1–3} Lipopolysaccharide (LPS), a common inflammatory agonist, is the biologically active constituent of endotoxin derived from the outer membrane of Gram-negative bacteria. Bacterial endotoxin has been recently recognized as a potential mediator of inflammatory responses in atherosclerosis.\textsuperscript{4}

NADPH oxidase appears to be the principal enzymatic source of superoxide radicals (O\textsubscript{2}^{-}) in the vascular wall.\textsuperscript{5–7} NADPH oxidase–mediated oxidative stress has been implicated in the activation of redox-sensitive transcription factors and vascular expression of inflammatory genes, including cytokines, chemokines, and cellular adhesion molecules, for example, vascular cell adhesion molecule-1 (VCAM-1).\textsuperscript{8–9} Increased surface expression of VCAM-1 is an early marker of endothelial activation, which contributes to monocyte infiltration and vascular remodeling in the initial stages of atherosclerosis.\textsuperscript{10–11}

The catalytic subunit of NADPH oxidase is a membrane-bound protein called cytochrome b\textsubscript{558}, which comprises two subunits, NOX and p22\textsuperscript{phox}. These two catalytic subunits associate with several cytoplasmic regulatory subunits, viz., Rac1, p40\textsuperscript{phox}, p47\textsuperscript{phox}, p67\textsuperscript{phox}, p41\textsuperscript{nox}, or p51\textsuperscript{nox}. Several homologues of NOX, called NOX1–5, have been identified in different cell types.\textsuperscript{12–15} NOX2, which is also known as gp91\textsuperscript{phox}, is found in phagocytic cells, such as neutrophils and monocytes. Vascular endothelial cells contain NOX1, 2, and 4, and vascular smooth muscle cells NOX1, 4, and 5.\textsuperscript{14,15} Although it is possible that additional, as of yet unidentified NADPH oxidase subunits exist, it is well established that all NOX-containing enzymes require p22\textsuperscript{phox} for catalytic activity, because p22\textsuperscript{phox} serves as the docking protein for the other subunits and stabilizes the NOX subunit.\textsuperscript{16} Furthermore,
it has been shown that each molecule of cytochrome $b_{558}$ contains 2 molecules of heme. The 2 heme-bound iron ions are essential for electron transfer from NADPH to oxygen and, hence, $O_2^-$ generation by the enzyme. Removing iron from heme by heme oxygenase-1 (HO-1), or blocking heme synthesis, lowers NADPH oxidase activity because of destabilization and degradation of cytochrome $b_{558}$.

Therefore, there is a close relationship between cellular iron status and NADPH oxidase activity. Accordingly, the activity of NADPH oxidase was found to be significantly lower in patients with iron-deficient anemia, and iron supplementation increased—and eventually normalized—NADPH oxidase activity. In addition, several studies have observed an increased iron level in human atherosclerotic plaque.

Exactly how iron regulates NADPH oxidase and its subunits in arterial inflammation and atherosclerosis has not been established, and the role of iron in atherosclerosis remains controversial.

We have recently reported that the iron chelator, desferrioxamine (DFO), suppresses NADPH oxidase-mediated oxidative stress and VCAM-1 expression in an in vivo model of LPS-induced inflammation. In the present study, we sought to investigate the mechanisms by which iron enhances, and DFO suppresses, NADPH oxidase activity in human aortic endothelial cells (HAECs).

**Methods**

**Endothelial Cell Culture**

HAECs obtained from Cambrex Bio Science were cultured in endothelial cell growth medium at 37°C in a humidified 95% air-5% CO$_2$ atmosphere. Cells were harvested at confluence with 0.05% trypsin-0.02% EDTA (Cambrex Bio Science) and plated at a split ratio of 1:3. For experiments, passage 5 to 8 cells were grown to confluence in 96-well plates or 100-mm Petri dishes, using an endothelial culture medium consisting of M199 medium (Sigma) supplemented with 20% fetal bovine serum (FBS, Life Technologies), 100 ng/mL streptomycin, 100 IU/mL penicillin, 250 ng/mL fungizone, 1 mM/L glutamine (Sigma), and 1 ng/mL recombinant basic fibroblast growth factor (Roche).

Cell viability was assessed by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) cell proliferation and viability assay kit (R&D Systems).

**NADPH Oxidase Activity**

Cells were washed with ice-cold Hanks’ balanced salt solution (HBSS) and transferred to lysis buffer (Cell Signaling). The cell lysates were centrifuged for 10 minutes at 12,000g and transferred to lysis buffer (Cell Signaling). The cell lysates were centrifuged for 10 minutes at 12,000g and 4°C, and 20 μL of the supernatant was subjected to protein analysis. NADPH oxidase activity was assessed by measuring $O_2^-$ production in the presence of the substrate. NADPH (100 μmol/L, Sigma) as lucigenin-enhanced chemiluminescence (5 μmol/L lucigenin, Sigma). No enzymatic activity could be detected in the absence of NADPH. Reactions were initiated by the addition of 10 to 20 μL cell lysate containing 25 to 50 μg extracted protein. NADPH oxidase activity was expressed as relative light units (RLU)/min/mg protein.

**Western Blot Analysis of p22$^{phox}$, p47$^{phox}$, and Heme Oxygenase-1**

Equal amounts of protein (30 μg in 20 μL lysis buffer) were electrophoresed on 12% SDS polyacrylamide gels (Invitrogen), electro-transferred to a PVDF membrane (Invitrogen), and blotted with primary antibodies against p22$^{phox}$ (1:1000, a kind gift from Dr Frans B. Wientjes, University College London, UK; or 1:200, Santa Cruz), p47$^{phox}$ (1:200, Santa Cruz), or HO-1 (1:250, Stressgen). The secondary antibodies used were goat antirabbit IgG (1:2000) for p22$^{phox}$ and p47$^{phox}$, and goat antimouse IgG (1:2000) for HO-1. Blots were developed using ECL plus reagent (Amersham Biosciences). Pre-stained protein markers (Bio-Rad Laboratories) were used for molecular mass determination. To confirm equal protein loading, PVDF membranes were stripped and blotted with an antibody against actin. Molecular band intensity was determined by densitometry using NIH Scion image software.

**Total Cellular Iron Content**

Cells were transferred to lysis buffer (Cell Signaling), the lysates were centrifuged for 10 minutes at 12,000g and 4°C, and 20 μL of the supernatant was subjected to protein analysis (BCA kit, Bio-Rad). The remaining sample was digested in 69% nitric acid overnight at room temperature. Subsequently, the samples were diluted 1:100 in 1% nitric acid and subjected to a total iron assay using inductively coupled plasma-optical emission spectroscopy (ICP-OES). ICP-OES was also used to measure the total iron content in M199 containing 20% FBS. The standard curve was prepared using a standardized iron solution (ICP-026, Ultra Scientific).

**Cellular Heme Level**

Cells were transferred to lysis buffer (Cell Signaling), the lysates were centrifuged for 10 minutes at 12,000g and 4°C, and 20 μL of the supernatant was subjected to protein analysis (BCA kit, Bio-Rad). The remaining sample was transferred to a glass tube and boiled in 2 mol/L oxalic acid (Sigma) for 30 minutes to release iron from heme, generating protoporphyrin IX, which was measured using a fluorescence microplate reader (Spectra Max Gemini XS, Molecular Devices, excitation wavelength 410 nm, emission wavelength 600 nm).

**Real-Time Quantitative Polymerase Chain Reaction**

Quantification of human p22$^{phox}$, p47$^{phox}$, NOX4, and GAPDH was performed by amplification of cellular cDNA, using a DNA Engine Opticon 2 System real-time thermocycler (Bio-Rad). Optimized amplification conditions were: 300 mmol/L primers (Invitrogen) for p22$^{phox}$, p47$^{phox}$, NOX4, and GAPDH; 3 mmol/L MgCl$_2$; and annealing at 58°C. Copy numbers were calculated by the instrument software from standard curves generated from human p22$^{phox}$, p47$^{phox}$, NOX4, and GAPDH templates. The primer sequences used were as follows: (1) p22$^{phox}$ F: 5'-GGCTGGGTCGGCCTCTATGATCACA3'; R: 5'-AGCCCTACAGGCGCAGATGTTGAG3'; (2) p47$^{phox}$ F: 5'-TTT-GAGAAGGCCTCGTGCACC3'; R: 5'-CTGCTAAACACCTTGG-GAGCT3'; (3) NOX4 F: 5'-CAGAAGTTCGAAGCGTG3'; R: 5'-GGTGGAGGCATTACCA3'; (4) GAPDH F: 5'-GAAAGTGAAGGCTCCGGAGT3'; R: 5'-GAAGATGCGTGATGAG3'.

**Statistical Analysis**

Data are expressed as mean±SEM. Student unpaired t test or factorial analysis of variance (ANOVA) was used for statistical analysis of the original data. Significance was accepted at $P<0.05$.

**Results**

DFO Inhibits the LPS-Induced Increase in NADPH Oxidase Activity in Human Aortic Endothelial Cells

Incubation of HAECs for 18 hours with ≥1 μg/mL LPS (Escherichia coli 055:B5, detoxified) significantly increased NADPH oxidase activity, whereas no increase was observed with 0.2 μg/mL LPS (Figure 1a). None of the LPS concentrations used (0.2 to 10 μg/mL) caused a significant decrease...
in cell viability after 24 hours of incubation, as assessed by the MTT assay (data not shown).

Exposure of HAECs to 5 μg/mL LPS significantly increased NADPH oxidase activity after 12 and 24 hours of incubation (Figure 1b). This LPS-induced increase in NADPH oxidase activity was eliminated by pretreatment of the cells for 24 hours with 100 μmol/L DFO (Figure 1b); or for 1 hour with 100 μmol/L of the NADPH oxidase inhibitor, apocynin (data not shown). Thus, LPS increases endothelial NADPH oxidase activity in a time- and dose-dependent manner, and iron appears to play a critical role in this process.

Treatment of HAECs for 24 hours with 100 U/mL tumor necrosis factor α (TNFa) also significantly increased NADPH oxidase activity ≈2-fold compared to untreated control cells, and this effect was abolished by DFO. In contrast, incubation of HAECs for 24 hours with 10 ng/mL interleukin-1β did not affect NADPH oxidase activity, either in the absence or presence of DFO (data not shown).

DFO Inhibits the LPS or Iron-Induced Increase in Cellular Iron and Heme Levels

Incubation of HAECs with 5 μg/mL LPS for 24 hours significantly increased cellular iron and heme levels (Figure 2a and 2b, respectively). These effects of LPS were strongly suppressed by 24-hour pretreatment with 100 μmol/L DFO. Similarly, incubation of HAECs with 100 μmol/L ferric citrate for 48 hours significantly increased cellular iron and heme levels, which was inhibited by 45-hour coincubation with DFO (Figure 2a and 2b).

DFO Inhibits the LPS or Iron-Induced Increase in p22phox Protein and NADPH Oxidase Activity

Incubation of HAECs with 5 μg/mL LPS for 24 hours increased the protein level of p22phox, a heme-containing, catalytic subunit of NADPH oxidase (Figure 3a). The same observation was made in the human monocytic cell line, THP-1 (Figure 3b), which was used to confirm results obtained with HAECs. Incubation of HAECs or THP-1 cells with 100 μmol/L ferric citrate for 48 hours also increased
p22phox protein level were strongly inhibited by DFO (Figure 3a and 3b). Furthermore, LPS and iron increased NADPH oxidase activity in HAECs after 24 or 48 hours of incubation, respectively, which was blocked by DFO (Figure 3c, see also Figure 1b). Hence, the changes observed in NADPH oxidase activity in HAECs (Figure 3c) paralleled the corresponding changes in p22phox in the same cells (Figure 3a).

LPS and Iron Do Not Increase, but DFO Decreases, Heme Oxygenase-1 Protein, Whereas p47phox Protein Is Unaffected

Incubation of HAECs with iron or LPS did not significantly increase the protein level of HO-1 (Figure 4a and 4b). In contrast, treatment with DFO strongly suppressed HO-1, both in the absence and presence of iron or LPS (Figure 4a and 4b). Furthermore, the protein level of p47phox, a non heme-containing, regulatory subunit of NADPH oxidase, was not significantly affected by any of the treatments of HAECs, ie, LPS or iron without or with DFO (Figure 4a and 4c). These results are in striking contrast to the changes observed in the p22phox protein level (Figure 3a and 3b).

LPS Time-Dependently Increases Gene Expression of p22phox, Which Is Inhibited by DFO, but Has No Effect on p47phox and NOX4 Gene Expression

Incubation of HAECs with LPS for up to 24 hours, without or with 24-hour pretreatment with DFO, had no significant effect on the mRNA level of NOX4 (Figure 5a), which is the principal catalytic subunit of endothelial NADPH oxidase. The message level of p47phox also was not affected by LPS or DFO (Figure 5b), in agreement with the unchanged p47phox protein level (Figure 4a and 4c). However, LPS treatment time-dependently increased the mRNA level of p22phox, which reached a maximum after 3 hours of incubation and subsequently declined rapidly (Fig-
The LPS-induced increase in the p22phox mRNA level was eliminated by pretreatment of the cells with DFO (Figure 5c). These data support the notion that LPS increases NADPH oxidase activity in HAECs by upregulating gene transcription of p22phox, but not the other subunits of the enzyme, NOX4 and p47phox.

LPS Increases Cellular Iron and Heme Levels Only After 24 Hours of Incubation, Which Is Inhibited by DFO

Finally, the time-course of LPS-induced changes in cellular iron and heme levels was assessed. As shown in Figure 6, exposing HAECs to LPS did not affect iron and heme levels for up to 16 hours of incubation. However, significant increases were observed at 24 hours, which were blocked by pretreatment of the cells with DFO (Figure 6).

Discussion

Lipopolysaccharide-induced oxidative stress is causally related to increased NADPH oxidase activity; however, the underlying mechanisms are incompletely understood.4,26,31,32 The major findings of this study are that prolonged exposure of human aortic endothelial cells to LPS or iron increases cellular levels of iron, heme, and p22phox, a heme-containing, catalytic subunit of NADPH oxidase; and treatment of the cells with the iron chelator, desferroxamine, inhibits these effects and prevents the LPS or iron-induced increase in NADPH oxidase activity.

We found that LPS increased NADPH oxidase activity in HAECs in a time- and dose-dependent manner, which is consistent with the observation that LPS dose-dependently increased O$_2^-$ production in human blood vessels.33 Interestingly, our time-course studies showed that LPS caused a small, transient increase in NADPH oxidase activity within the first 30 minutes of incubation (see Figure 1b), likely attributable to activation of preexisting NADPH oxidase. However, this increase was not statistically significant and did not appear to be inhibited by DFO. A significant, sustained increase in NADPH oxidase activity was observed between 12 and 24 hours of incubation with LPS, which was abrogated by DFO. These findings are in agreement with published data that increased O$_2^-$ production by NADPH oxidase required prolonged exposure to LPS, both in vitro and in vivo.26,31,32

Prolonged exposure to LPS also increased cellular iron, heme, and p22phox protein levels in HAECs. As a possible explanation for these observations, LPS has been shown to upregulate the divalent metal transporter 1 (DMT1), an iron importer, in bronchial epithelial cells.34 Increased iron uptake supplies cellular iron for heme biosynthesis, which in turn may help stabilize the heme protein, p22phox.19,20 We also observed that TNFα increased NADPH oxidase activity in HAECs in a DFO-sensitive manner, and TNFα, like LPS, is known to upregulate DMT1 in bronchial epithelial cells.34 However, although DMT1 is abundant in HAECs, its level was not affected by incubation with LPS (data not shown).
Therefore, the mechanism by which LPS stimulates iron uptake into HAECs remains to be fully elucidated.

The above long-term effect of LPS on cellular iron and heme levels may explain why p22phox protein and NADPH oxidase activity were increased in HAECs after 24 hours of incubation. An additional, major role of iron in NADPH oxidase activity is indicated by the observation that DFO abrogated LPS-induced p22phox gene transcription, which peaked at around 3 hours of incubation with LPS. It is conceivable that cellular labile ("free") iron, for example, by increasing oxidative stress, plays a critical role in LPS or TNFα-induced activation of the redox-sensitive transcription factors, NFκB and AP-1, and subsequent p22phox gene expression. In contrast, LPS and DFO had no effect on p47phox and NOX4 gene expression, suggesting a different mechanism of transcriptional regulation independent of iron.

Incubating HAECs with excess iron, in the form of ferric citrate, mimicked the effects of LPS on cellular iron, heme, and p22phox protein levels. Ferric citrate was used because the majority of labile iron in humans is found as a complex of ferric iron with citrate. As discussed above, DFO inhibited the iron or LPS-induced changes in cellular iron, heme, p22phox, and NADPH oxidase. DFO is transported into cells via endocytosis and remains associated with endosomes, from which labile iron is transported to mitochondria for heme biosynthesis. Thus, DFO chelates free iron and blocks heme synthesis, which may explain why it affected the cellular level of the heme protein, p22phox, but not the nonheme protein, p47phox.

Neither iron nor LPS affected heme oxygenase-1 in HAECs. Induction of HO-1 has been shown to lower NADPH oxidase activity attributable to decreased heme availability and destabilization and degradation of p22phox and cytochrome b558. Hence, our findings presented here and elsewhere indicate that LPS and iron increase NADPH oxidase activity independently of HO-1, most likely by upregulating p22phox gene expression and stabilizing the protein by increasing cellular iron uptake and de novo synthesis of heme.

Interestingly, DFO blocked HO-1 expression irrespective of the addition of LPS or iron. These data suggest that there is a negative feedback loop between iron and HO-1: chelation of iron with DFO reduces the iron supply for synthesis of heme and stabilization of p22phox, and thus NADPH oxidase activity declines. In turn, this mechanism may negatively regulate HO-1 to prevent further degradation of heme and, hence, decreased NADPH oxidase activity. The iron content in HAEC growth media (M199 containing 20% FBS) is about 10 μmol/L, whereas up to 100 μmol/L ferric citrate was added in our experiments to induce an effect on p22phox and NADPH oxidase activity. Nevertheless, the low iron content in the media seems enough to strongly induce HO-1 expression in HAECs, because neither added iron nor LPS further increased HO-1.

In summary, our data show that prolonged exposure to LPS or iron increases endothelial NADPH oxidase activity, in parallel with increased p22phox gene transcription and increased cellular levels of iron, heme, and p22phox protein. All of these effects of LPS and iron were strongly inhibited by the iron chelator, desferrioxamine. Therefore, chelation of excess iron may help attenuate vascular oxidative stress and inflammation and inhibit the development of atherosclerotic vascular diseases.

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Disclosures
None.

References


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